Proliferation and Differentiation Biomarkers in Colorectal Mucosa and Their Application to Chemoprevention Studies

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Colorectal cancer is one of the leading causes of morbidity and mortality in Western countries. Because its prognosis is relatively unaffected by improvements in surgery and chemotherapy, increasing interest has recently been directed toward chemoprevention. Intermediate biomarkers of abnormal cell proliferation, differentiation, and gene expression have recently been identified and have served to measure effects of chemopreventive agents in rodent models and in short-term human clinical trials. Alterations in cell proliferation and differentiation have been found in preneoplastic diseases and in normal-appearing colorectal mucosa of patients at increased risk for malignancy. Several techniques are available for measuring these alterations, and standardization and comparison of different methods are underway to assess the utility of various intermediate biomarkers in chemoprevention studies.

Introduction

The potential importance of chemoprevention in populations at increased risk for colorectal cancer has been emphasized in recent years. This is partly because improvements in chemotherapy, radiotherapy, and surgery have not significantly changed the prognosis of colorectal cancer, and this malignancy is still one of the leading causes of morbidity and mortality in western countries (*1*). Epidemiologic studies and studies in rodent models have indicated a possible chemopreventive role for various natural substances and pharmacologic agents (*2,3*). However, their effect on prevention of human disease has generally not been clearly established, and large clinical trials in humans are need for definitive results.

One of the main problems in planning large, chemopreventive interventions in humans is that the development of malignant neoplasms is a slow, multistep process that usually takes many years. Moreover, the incidence of cancer in the general population is relatively low. Therefore, carrying out human chemopreventive intervention studies that decrease morbidity and mortality with neoplasia as an end point has been very time comsuming and costly. Insufficient subjects or short follow-up periods significantly decrease the power of these studies, making it impossible to detect real but small protective effects of chemopreventive agents.

Therefore, it appears to be essential to develop and validate intermediate biomarkers of abnormal cell growth and maturation for use in chemopreventive intervention studies. These biomarkers should be the result of actual events occurring the initiation and promotional phases of tumor development in order to be optimum candidates for modification by chemopreventive agents. Intermediate biomarkers with these characteristics can also be tested for their role in identifying populations at increased risk for cancer and in developing human screening programs with reasonable cost–benefit ratios (*4*).

Biomarkers of Increased Susceptibility to Gastrointestinal Cancer

Intermediate biomarkers of abnormal cell growth in human subjects have been developed through several investigative approaches. These include the identification of individuals or population groups at either increased or at low risk for neoplasia and the application of these groups to studies identifying biomarkers that can be modulated by nutritional and chemopreventive interventions.

Ideal intermediate biomarkers of abnormal cell growth that can function effectively will eventually have to be demonstrated to satisfy usual criteria of conventional laboratory tests (i.e., high specificity, high sensitivity, good positive and negative predictive value). Moreover, in a population at increased risk for cancer with abnormal expression of an intermediate biomarker, a change in expression of the biomarker in a normal direction by a chemopreventive intervention should reflect a reduced risk of neoplasia (*5*). Therefore, secondary events or "side effects" that

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Table 1. Expansion of the proliferative compartment of epithelial cells in studies of the human large intestine.

Disease	References
Familial polyposis	Deschner et al., 1963 (6)
	Deschner et al., 1975 (7)
	Lipkin et al., 1983 (8)
	Lipkin et al., 1984 (9)
Sporadic adenomas	Terpstra et al., 1987 (10)
	Risio et al., 1988, 1991 (11,12)
	Ponz de Leon et al., 1988 (13)
	Roncucci et al., 1991 (14)
	Scalmati et al., 1990 (15)
Colon cancer	Maskens et al., 1977 (14)
	Lipkin et al., 1983 (8)
	Lipkin et al., 1984 (9)
	Ponz de Leon et al., 1988 (13)
Ulcerative colitis	Serafini et al., 1981 (17)
	Deschner et al., 1983 (18)
	Biasco et al., 1990 (19)

may accompany the evolution of neoplasia are not applicable as biomarkers because they are not part of the neoplastic process. Thus, although anemia is a frequent finding patients with atrophic gastritis at increased risk for gastric cancer, reducing the anemia does not reduce the cancer risk.

Moreover, intermediate biomarkers should be reasonably easy to measure in individuals and in population groups; the measurements should be easy to standardize so the data obtained from different laboratories are comparable. The measurement techniques employed should also be sufficiently inexpensive and reliable to be used in screening programs or in intervetion trials involving many patients.

None of the biomarkers currently being studied have been shown to fulfill all these criteria, but studies are now underway to attempt to validate several intermediate biomarkers of abnormal cell proliferation, differentiation and gene expression; in some of these studies chemopreventive agents have also been given to subjects in high-risk populations to evaluate their effects on the biomarkers.

Biomarkers of Cell Proliferation

The incorporation of labeled DNA precursors by cells in S phase of the proliferative cycle is an effective and reliable way to measure cell proliferation in renewing tissue (4). [³H]Deoxythymidine (dThd) has been used fairly extensively in studies of cell proliferation in human and rodent tissues, and it has been possible to define normal numbers of cells and spatial patterns of proliferating and differentiating cells in renewing mammalian tissues and alterations in the cells and tissues of patients at increased risk for neoplasia. With these measurements, patterns of cell proliferation have been shown to be modified in several groups of human subjects previously identified as being at increased risk for gastrointestinal cancer and in rodent models treated with chemical carcinogens. Bromodeoxyuridine (BrdU), incorporated into the DNA of cycling cells as is [³H]dThd, can be detected with immunoperoxidase technique; the results obtained with BrdU and (³H]dThd have generally been comparable after pulse injections.

Several diseases that affect individuals at increased risk for colorectal cancer have been identified in which the size of the proliferative compartment has increased compared to low-risk subjects (Table 1). Familial polyposis is an inherited, autosomal dominant disease characterized by a colon lined with adenomatous polyps and the development of colorectal cancer at a very early age; several extracolonic manifestations can also be variably present in affected members of the kindreds. The normal-appearing rectal mucosa of the affected patients shows a pattern of cell proliferation that clearly differs from that of normal people. The proliferative compartment of normal colorectal mucosa of individuals at low risk for colorectal cancer, and of normal rodents, is located in the lower part of the colonic crypts. The total labeling index (i.e., labeled cells versus total cells) of low-risk controls and familial polyposis patients does not differ significantly, but the patients show a shift of the proliferative compartment toward the top of the crypts. Thus, the main proliferative alteration noted in the colonic mucosa of these patients is not an increase in overall cell proliferation labeling index, but a lumenward displacement of the zone of active cell proliferation in the crypts [see, for example, Deschner et al. (6,7) and Lipkin et al. (8,9)].

The polyp or dysplasia cancer sequence is a generally accepted hypothesis related to the pathogenesis of colon cancer (20,21), and patterns of cell proliferation in patients with adenomatous polyps have been widely studied. It has been reported that an expansion of the proliferative compartment is present along the entire colon in individuals at increased risk, no matter where the lesion is (10,13,14). A correlation between size and degree of dysplasia in adenomas and degree of alteration of cell proliferation also has been noted (12). The total labeling index of patients with polyps or cancer has been reported to be significantly higher than that of normal controls using [³H]dThd or BrdU in some studies (8,11), and in others cell proliferation in the upper crypt significantly increased, with corresponding expansion in size of the proliferative compartment (12,13,16).

Patients with previous surgery for colorectal cancer are known to be at increased risk for a second malignancy; the mucosa of remaining colon has shown increased overall cell proliferation and an increased proliferative compartment size in patients with recurrent polyps compared to nonrecurrent (15), suggesting an association between increased cell proliferation and risk of methachronous lesions. Chronic ulcerative colitis is a disease characterized by a defined risk of colorectal cancer that is further correlated to the extent and duration of the disease. A shift of the proliferative compartment towards the top of the crypt has been found in the normal-appearing mucosa of patients with chronic ulcerative colitis (17-19).

Several other methods have been used to measure cell proliferation. Ki67 (22) and proliferating cell nuclear antigen (PCNA) (23) are markers identified in cycling cells, detectable by immunohistochemistry, but their patterns of expression in healthy mucosa and especially in disease have not yet been clearly defined. The metabolism of polyamines and particularly levels of a key enzyme, ornithine decarboxylase (24), have been related to cell proliferation, but their biochemical determination in the tissue can only suggest total proliferative activity, and a minor altered pattern may be undetectable if it is not accompanied by an increase in the total labeling index. A similar problem can

Table 2. Biomarkers of abnormal differentiation of gastrointestinal epithelial cells of the large intestine.

Biomarker	References
Alterated mucin profiles	Allen et al., 1988 (26)
	Sugihara et al., 1987 (27)
Altered patterns of cyto-skeletal proteins	Carboni et al., 1987 (28)
	Stallmach et al., 1990 (29)
Blood-group antigens	Kim et al., 1986 (30)
	Itzkowitz et al., 1990 (31)
	Yan et al., 1990 (32)
Modified lectin-binding pattern	Rhodes et al., 1988 (33)
	Sams et al., 1990 (34)

arise for measurement of the percentage of cells in S phase determined by cytofluorimetry (25); this analysis also can only give an idea about the total labeling index without any distinction of cell proliferation in different parts of the crypt.

Biomarkers of Cell Differentiation

The studies listed in Table 2 are only a selected list with a few illustrations of the topic of cell differentiation biomarkers. Cell proliferation measurements are now gaining increased complexity (with new groups of PCNA molecules associated with cell cycle functions), and cell differentiation biomarkers are similarly becoming more complex. Multiple measurements are now available to distinguish cycling cells from quiescent proliferative

Table 3. Effect of chemopreventive agents on biomarkers of cell proliferation and differentiation in humans.

Agent	Effect	References
Calcium	Decreased hyperproliferation in patients with familial colon cancer (1250 mg daily for 3 months)	Lipkin et al., 1989 (37)
	Decreased hyperproliferation in patients at increased risk (1250 mg daily for 2 months)	Rozen et al., 1989 (38)
	Decreased hyperproliferation in patients at increased risk (2000 mg daily for 3 months)	Lynch et al., 1991 (39)
	Increased SBA binding	Yang et al., 1991 (40)
Fiber	Wheat bran (13.5 g fiber) decreased rectal cell proliferation	Alberts et al., 1990 (41)
Vitamins	Ascorbic acid (3g daily for 18 months) decreased cell proliferation in FAP patients	Bussey et al., 1982 (42)
	Betacarotine (20 mg daily for 2 years) contracted the zone of proliferation toward the crypt base in patients with polyps	Macrae et al., 1991 (43)
	Vitamin A (30,000 U), vitamin C (1 g) and vitamin E (70 mg) (daily for 6 months) reduced size of proliferative compartment in flat rectal mucosa in patients with adenomas	Paganelli et al., 1991 (44)
ω-3 Fatty acids	ω-3 Fatty acids reduced size of proliferative compartment in flat mucosa of patients with colorectal adenomas	Anti et al., 1991 (45)

Abbreviations: SBA, soybean agglutinin; FAP, familial adenomatous polyposis.

cells, degrees of cell differentiation, and late-stage events in the cell's life cycle. In normal adult colorectal mucosa, which contains cells at all stages of differentiation, some currently studied cell components include cytoskeletal elements, glycoproteins, modified blood-group antigens, and newer measurements of modified gene expression.

Among recent studies on modified properties of differentiating cells, it has been proposed that lack of expression of O-acylated sialomucins in epithelial cells in chronic ulcerative colitis is related to dysplasia and that it can serve as a biomarker of progression to malignancy (26). However, a lack of O-acetyl substituted sialic acid does not seem to be a marker of familial adenomatous polyposis, a condition also associated with increased cancer risk (27).

Among cell components that develop during their normal and abnormal differentiation, cytokeratins and other intermediate filaments become modified; it has been shown that their compositions and distributions vary in different cell types during different stages of cell differentiation and in disease states. Thus, the distribution of the brush-border-specific microvillar protein villin, in human colonic mucosa detected by immunohistochemistry, shows an altered pattern in dysplastic and neoplastic disease (28). Further, different proteins that bind basement membrane glycoprotein laminin have been found in preneoplastic compared to normal mucosa (29).

Carbohydrate blood group-related antigens are also expressed in colonic epithelial cells and show two kinds of alterations in preneoplastic and neoplastic tissues (31). The first type involves the synthesis of new epitopes not expressed by normal mucosa, usually accomplished by a process of oligosaccharide elongation. An example of this is the expression of the trifucosyl or extended LeY antigen in adenomas and cancer cells but not in normal mucosa (30). The second kind of alteration observed is the unmasking and subsequent expression of normally cryptic antigens following incomplete glycosylation (32). These antigens are not expressed by normal colonocytes, but they are expressed in premalignant colonic tissue such as dysplastic adenomatous polyps and chronic ulcerative colitis with dysplasia.

Lectin-binding of cell surface carbohydrate residues becomes modified with the differentiation of normal epithelial cells and in stages of development of precancerous diseases. Abnormal patterns of lectin binding have been described in the colorectal mucosa of patients with inflammatory diseases or adenomas (33,34).

Further Aspects of the Molecular Biology of Colon Cancer

Oncogene mutations, the overexpression, deletion, and mutation of tumor-suppressor genes, DNA hypomethylation, and other genetic alterations have now been identified in preneoplastic and neoplastic lesions in the colon. The familial adenomatous polyposis gene has been located on chromosome 5, and a recently mapped point mutation and loss of p53 on chromosome 17p has been found in some lesions. DCC and MCC genes have been described, and myc also may be involved (35,36). Among many reviews of this topic, several points are relevant for chemoprevention studies.

Having available genetic probes for the disease would naturally help define populations at risk, and detect individuals who could

benefit most from closer follow-up and intervention. However, none of the genetic alterations described to date can be used with certainty to detect most or all individuals at risk. Human cancers appear to have a multistep evolution, and many environmental factors are involved. Attempts to modulate or suppress oncogene expressions have begun and are likely to increase in the future.

Therefore, it would appear that information on the location, structure, and function of the genes involved in colorectal cancer will be useful in selecting individuals for screening and for chemopreventive interventions, and modifications that are induced in gene structure and expression may prove to be useful in assessing the efficacy of interventions. Following the multistep concept of cancer etiology, chemopreventions might in the future be able to inhibit initiation or promotion events in cells genetically predisposed to cancer. The expression of biomarkers of cell differentiation and proliferation may similarly be useful measurements because they are theoretically modifiable.

Modulation of Cell Proliferation and Differentiation Biomarkers by Chemopreventive Interventions

A few selected examples of chemopreventive interventions studied in humans are given in Table 3. Fiber, calcium, vitamins, and ω-3 fatty acids are all constituents of human diets, and various epidemiologic and experimental findings support protective roles for these constutuents against colorectal cancer (3,4). Results obtained with fiber have differed depending on types and amounts given in various studies (46). Calcium has shown more consistent and reproducible results in most animal and human studies, and calcium supplementation in the diet in most studies has reduced expansions of the proliferative compartment in patients with familial colon cancer predisposition and sporadic adenomas (37–39). Calcium is unable to further decrease an already low level of proliferation (37). In one study calcium also was shown to change soybean agglutinin lectin binding, increasing it toward a pattern more typical of well-differentiated cells (40). Results obtained with vitamins (42–44) and ω-3 fatty acids (45) are also of interest, and measurements have begun in a number of studies.

Conclusions

Intermediate biomarkers of cell proliferation have generally been reliable and quantifiable, present in various populations at risk, and modifiable by chemopreventive agents. However, differences detected are often very small, and careful evaluations are mandatory, including measurements of size of proliferative compartments to detect effects of agents.

New measurements of the stages of cell differentiation are increasing and are beginning to be quantitated. Assessments of cell proliferation, differentiation, and gene structure and expression with intermediate biomarkers are currently being carried out to assess their utility and to detect more subtle changes in the cells in chemoprevention studies.

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